PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/00, 5/00, 1/20 C12N 1/00, A01H 5/00	A1	(11) International Publication Number: WO 91/02059 (43) International Publication Date: 21 February 1991 (21.02.91)

(21) International Application Number: PCT/US90/04281

(22) International Filing Date: 31 July 1990 (31.07.90)

(30) Priority data:
387,739 1 August 1989 (01.08.89) US

(71) Applicant: PIONEER HI-BRED INTERNATIONAL, INC. [US/US]; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US).

(72) Inventors: BOWEN, Benjamin, A. ; 3008 36th Street, Des Moines, IA 50310 (US). BEACH, Larry, R. ; 3939 Maquoketa, Des Moines, IA 50311 (US). WESSLER, Susan ; 320 Idylwood Drive, Athens, GA 30605 (US). LUDWIG, Stephen, R. ; 138 Ashley Circle, Athens, GA 30605 (US).

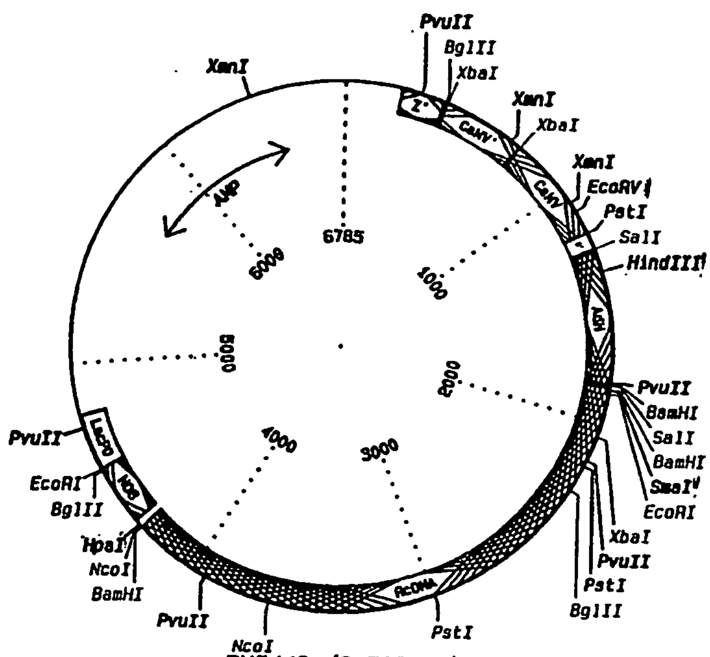
(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: TRANSCRIPTIONAL ACTIVATORS OF ANTHOCYANIN BIOSYNTHESIS AS VISUAL MARKERS FOR PLANT TRANSFORMATION

(57) Abstract

Transcriptional activators of anthocyanin biosynthesis, operatively linked to a suitable promoter in an expression cassette have widespread utility as non-phytotoxic markers for plant cell transformation. A clone encoding for one of these genes (for example, the Lc gene or a translational equivalent thereof in maize) can be fused to appropriate expression sequences to provide an expression cassette which can be introduced into plant cells by any desired transformation method. This also provides materials and methods for production of novel ornamental plants having anthocyanin pigmentation.



pPHI443 (6.785 kb)

* See back of page

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MC	Monaco
AU	Australia	FI	Finland	MG	Madagascar
BB	Barbados	FR	France	ML	Mali
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Fasso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GR	Greece	NL	Netherlands
BJ	Benin	HU	Hungary	NO	Norway
BR	Brazil	IT	Italy	PL	Poland
CA	Canada	JP	Japan	RO	Romania
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
DE	Germany	LK	Sri Lanka	TD	Chad
DK	Denmark	LU	Luxembourg	TG	Togo
				US	United States of America

TRANSCRIPTIONAL ACTIVATORS OF ANTHOCYANIN BIOSYNTHESIS AS VISUAL MARKERS FOR PLANT TRANSFORMATION

Technical Field

This invention relates to the use of anthocyanin pigmentation as a non-phytotoxic marker for transformation of plant cells.

In the transformation of plant cells to incorporate new DNA sequences, it is frequently the case that the DNA sequence which is being incorporated into the plant cell genome codes for a protein which does not produce an immediately identifiable result. For example, a gene which codes for the insecticidal toxin of Bacillus thuringiensis (BT) does not produce any immediately identifiable change in the characteristics of cells transformed by that gene, unless the cells are destructively analyzed for their content of BT toxin. Likewise, in the case of genes affecting complex multigenic traits, their ultimate effect on characteristics such as plant height, resistance to stalk lodging, and other factors cannot immediately be assessed in vitro. Thus, it is desirable to incorporate into the DNA sequence which is inserted into the plant cells for transformation a marker gene which provides an easy way of identifying and separating transformed cells.

One technique which has been used is to incorporate into the DNA sequence which is introduced into the plant cells a gene which codes for a protein, such as an enzyme, which confers resistance to an antibiotic or herbicide. Then, when the plant cells are cultured in a medium containing the selected herbicide or antibiotic, non-transformed cells are selectively killed by the antibiotic or herbicide, leaving only transformed cells. However, in some cases it will not be desirable to incorporate into a particular plant species resistance to an antibiotic or herbicide. In other cases, it will not be desirable to

identify transformed cells destructively, i.e., it will be important to retain viable transformed cells as a result of the transformation process. In yet other cases it will be desirable to follow transformed tissues on an ongoing basis, without the need for repeated assays of enzyme activity. Accordingly, there is a continuing need for non-phytotoxic marker genes which easily and reliably serve to identify transformants among a population of plant cells subjected to a transformation.

Brief Description of the Drawing

Fig. 1 shows a typical dose response curve in maize using the expression cassette of this invention.

Fig. 2 shows a plasmid map of pPHI443.

Fig. 3 shows the sequence of the Lc cDNA from maize.

Disclosure of the Invention

Many plant cell types, including most maize cell types have the capacity to accumulate red anthocyanin pigments. In the following discussion, maize plants will be used as the prototype for all plant cell types which can accumulate anthocyanin pigments, since they represent a species which is both agronomically highly significant and relatively difficult to transform reliably. However, it is to be understood that all plant cells which are capable of accumulating anthocyanin under the control of transcriptional activator genes are encompassed within the scope of this invention.

At least five genes in maize are known to encode enzymes which are required for the synthesis of anthocyanin pigments. Several other loci, including R, B, and Lc, determine the pattern and timing of anthocyanin biosynthesis in the maize plant and seed. The expression of these regulatory genes is complex. For example, more than 50 naturally occurring alleles of R that condition unique

patterns of pigmentation have been described. One allele, R-nj, has been cloned by tagging with the transposable element Ac. R-nj is approximately 90% homologous with the R genes P, S, Lc and B and has been used to isolate an Lc cDNA clone. The protein encoded by the Lc cDNA has been shown to have features characteristic of a transcriptional activator. From these data and previous genetic analyses, it has been determined that all R genes encode functionally equivalent proteins and that developmental specificity of pigmentation is determined by differences in the R promoter regions. Thus, it has now been determined that transcriptional activators of anthocyanin biosynthesis, operatively linked to a suitable promoter in an expression cassette, have widespread utility as non-phytotoxic markers for plant cell transformation. Accordingly, a clone encoding for one of these genes (for example, the Lc gene or a translational equivalent thereof in maize) can be fused to appropriate expression sequences to provide an expression cassette which can be introduced into plant cells by any desired transformation method, such as microprojectile bombardment. Red cells accumulating anthocyanin can be readily detected in epidermal and sub-epidermal layers of most tissues tested. Since this expression, indicated by accumulation of anthocyanin, can be followed in living tissue, the expression cassettes of this invention, comprising a genomic or cDNA clone ("clone") or translational equivalent thereof coding for a plant transcriptional activator gene for anthocyanin biosynthesis operably linked to plant regulatory sequences which cause expression of the clone in plant cells, provides a useful reporter/marker gene and transformation vector for maize and other plant cells. The ability to follow expression in living tissue by use of a non-toxic marker provides the ability to select cell lineages which can give rise to stably transformed plants.

In addition, this expression cassette can be incorporated into a bacterial transformation vector which causes expression or replication of the expression cassette in living bacterial cells as an intermediate step prior to introduction into plant cells.

The resulting bacterial vectors can be readily inserted bacteria for expression and characterization of the sequence. Accordingly, the present invention also provides bacterial cells containing as a foreign plasmid at least one copy of the foregoing bacterial expression vector. In one preferred embodiment, the plant expression cassette preferably includes a strong constitutive promoter sequence at one end to cause the gene to be transcribed at a high frequency, and a poly-A recognition sequence at the other end for proper processing and transport of the messenger RNA. An example of such a preferred (empty) expression cassette into which the cDNA of the Lc gene can be inserted is the pPHI414 plasmid developed by Beach, et al. of Pioneer Hi-Bred International, Inc., Johnston, Iowa. In other cases, tissue-specific promoters can be employed to monitor transformation in selected tissues of the plant. An example of a tissue-specific promoter is the widely used RuBP carboxylase small subunit promoter. Highly preferred plant expression cassettes will be designed to include one or more structural genes conferring the desired transformation trait, such as insect resistance or increased yield. It is important that the cloned Lc gene have a start codon in the correct reading frame for the structural sequence. The plant expression vectors of this invention can be introduced into plant cells using any convenient technique, including electroporation (in protoplasts), microprojectile bombardment, and microinjection, into cells from monocotyledonous or dicotyledonous plants, in cell or tissue culture, to provide transformed plant cells containing as foreign DNA at least one copy of the DNA sequence of the

plant expression cassette. Preferably, the monocotyledonous species will be selected from maize, sorghum, triticale, wheat and rice, and the dicotyledonous species will be selected from soybean, alfalfa, tobacco, canola and tomato. Using known techniques, protoplasts can be regenerated and cell or tissue cultures can be regenerated to form whole fertile plants which carry and express the desired marker gene. Accordingly, a highly preferred embodiment of the present invention is a transformed maize plant, the cells of which contain as foreign DNA at least one copy of the DNA sequence of an expression cassette of this invention. Finally, this invention provides transformation methods which use anthocyanin as a non-phytotoxic marker, comprising the steps of:

a) culturing cells or tissues from a selected target plant;

b) introducing into the cells the cell or tissue culture at least one copy of an expression cassette comprising a sequence coding for a plant transcriptional activator for anthocyanin biosynthesis, operably linked to plant regulatory sequences which cause the expression of the sequence in the cells, and

c) identifying transformed cells by their anthocyanin pigmentation. The sequence can be a genomic or cDNA clone, or a sequence which is translationally equivalent to a genomic or cDNA clone for the transcriptional activator. By "translationally equivalent" is meant that the DNA sequence in proper reading frame forms codons which translate to an amino acid sequence which is functionally equivalent to the amino acid sequence of the transcriptional activator produced by the genomic or cDNA clone, or is complementary (using normal C-G and A-T pairing) to a DNA sequence which in proper reading frame forms codons which translate to an amino acid sequence which is functionally equivalent to the amino acid sequence of the transcriptional activator

produced by the genomic or cDNA clone. Translation tables which show codon equivalences are found in most textbooks of molecular biology. It will be apparent that this marker gene, which uses pigmentation to provide visual evidence of transformation in plant cells, readily lends itself to use in combination with any of a variety of cell sorters which are capable of sorting cells on the basis of color, since accumulation of anthocyanin imparts a distinctive red or purple color to transformed cells. Thus, this invention also provides a method as described above, in which transformed cells are additionally separated from non-transformed cells by a cell sorter which sorts on the basis of color.

It will also be appreciated by those of ordinary skill that the plant vectors provided herein can be incorporated into Agrobacterium tumefaciens, which can then be used to transfer the vector into susceptible plant cells, primarily from dicotyledonous species. Thus, this invention provides a method for transformation of Agrobacterium tumefaciens-susceptible dicotyledonous plants in which the expression cassette is introduced into the cells by infecting the cells with an Agrobacterium tumefaciens, a plasmid of which has been modified to include the expression cassette provided herein. Since the marker expression cassette provided by this invention causes accumulation of a naturally occurring pigment, no enzyme assay or substrate is needed to determine activity of the gene.

The expression cassettes of this invention, because they employ transcriptional activator sequences for anthocyanin biosynthesis, have many potential applications in studying the molecular genetics and developmental biology of plants such as maize. For example, domains of the R protein thought to be important for DNA binding and transcriptional activation can be altered by site-directed mutagenesis of the Lc cDNA coding sequence in pPHI443. For

reference, the sequence of maize Lc cDNA is shown in Figure 2. By reintroducing mutant constructs into maize tissues of the appropriate genotype, the contribution of these domains to R function can be addressed. The Lc cDNA can also be used as a reporter gene for the analysis of promoters and other cis-acting control regions in maize. Unlike other reporter genes currently in use, such as CAT, GUS, and NPTII, no complicated biochemical assay or histochemical staining procedure is required for its detection. Pigmented cells are visible as early as 14 hrs after bombardment, and expression is easily quantitated by counting the number of red cells seen in a fixed number of bombardments. In addition, red cells retain their color in tissues treated with GUS histochemical stain. This means that gene expression from two reporter constructs can be visually monitored in the same tissue.

EXAMPLE 1

EXPRESSION CASSETTE

Clones of the Lc gene were obtained from Ludwig, Wessler et al. at the University of Georgia, who isolated them as follows:

For the 3.7 Kb HindIII fragment, total genomic DNA was digested to completion with HindIII, ligated to HindIII digested Charon 35 arms, packaged in vitro and plated on K803 cells. Of 5 x 10^5 phage screened with the pR-nj:1 insert, one recombinant phage contained the 3.7 kb HindIII genomic fragment. A cDNA library in lambda gt10 was constructed using a Pharmacia cDNA kit and 5 ug poly(A)$^+$ RNA from female spikelets isolated 10 dap. The library was screened with the 3.7 kb HindIII genomic Lc clone using standard methods, as described in Maniatis, T., Fritsch, F., and Sambrook, J., (1982) Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor New York). Of 3 x 10^5 recombinant phage screened with the

3.7 kb HindIII fragment, one phage contained a 2.5 kb insert. The 6 kb HindII genomic fragment was isolated by ligating 5 to 7 kb HindIII genomic fragments into the SpeI site of lambda Zap (Stratagene), packaged and plated. Of 2 x 10^5 recombinant phage screened with the 3' end of the cDNA (position 880-1772), twelve phage contained the 6 kb HindIII fragment.

Genomic and cDNA inserts were subcloned into pUC119. Overlapping subclones and unidirectional deletion clones were isolated and sequenced by the dideoxy method of Sanger et al., Proc. Natl. Acad. Sci. USA, 74, 5463-5467 (1977).

The start of transcription was determined by primer extension using the method of Dunsmuir et al., Plant Molecular Biology Manual (Kluwer Academic Publishers, Hingham, Massachussetts) Chapter C1, pp. 1-17. Five ug of poly(A)$^+$RNA and 0.1 pmol (7.5 x 10^5 cpm) of the ^{32}P-labelled oligonucleotide 5' CGTGAACCGGCGGACGAGGG 3'were hybridized at 55°C for 3 hrs. The primer was extended for 45 min at 37°C with AMV reverse transcriptase. RNase protection experiments were performed according to Promega Biotech. A 1.6 kb HindIII-SphI Lc genomic fragment was subcloned into pGEm-4Z (Promega). The plasmid (p259) was linearized with HindIII and transcribed in vitro with SP6 polymerase. The labelled RNA (1.5 x 10^5 cpm) was added to 5 ug poly(A)$^+$RNA isolated from female spikelets and hybridized overnight at 45°C. The unhybridized RNA was digested for 1 hr at 30°C with 40 ug/mL RNase A and 8 U/mL RNase T_1. The primer-extended and RNase protection products were each separated on an 8% acrylamide sequencing gel. The cDNA clone was sub-cloned into pGEM-7Z (Promega). This plasmid (p266) was linearized with XhoI and *in vitro* transcribed with SP6 polymerase according to the manufacturer (Promega). The RNA was in vitro translated for 60 min in the presence of [^{35}S]-methionine using the rabbit reticulocyte lysate system (Promega). The protein products were separated on a 10%

Laemmli gel. The sequence of the cDNA clone was identical to sequences found in the two genomic fragments confirming that the cDNA was derived from Lc. The transcription unit was found to span approximately 7 kb with introns at positions 228-229, 387-388, 648-649, 745-746, 760-761, 817-818, 1566-1567, and 1996-1997. All introns contained the consensus splice junction sequence (5' GT-- -- AG 3'). Primer extension was used to determine the start of transcription. A 20-base oligonucleotide was hybridized to poly(A)$^+$RNA isolated from plants with and without Lc and extended with reverse transcriptase. Two major bands were observed which were used to define nucleotide positions 1 and 3. An RNase protection experiment confirmed this as the start of transcription. The 5' end of the Lc cDNA starts 20 bp from the transcription start site.

The cDNA sequence contains a 610 amino acid open reading frame beginning with an AUG at nucleotide position 236 and ending with a stop codon at nucleotide position 2066.

The plasmid pPHI443 is a pUC18 plasmid containing an enhanced 35S promoter spanning nucleotides - 421 to +2 of Cauliflower Mosaic Virus with the region from - 421 to - 90 duplicated in tandem, a 79 bp HindIII SalI fragment from pJII101 spanning the 5' leader sequence of Tobacco Mosaic Virus, a 579 bp fragment spanning the first intron from maize Adh1-S, a 2415 bp XbaI fragment spanning the Lc cDNA, and a 281 bp fragment spanning the polyadenylation site from the nopaline synthase gene in pTiT37. The control plasmid pPHI459 contained identical expression signals, but an 1870 bp fragment from pRAJ275 spanning the beta-D-glucuronidase (GUS) coding sequence which was inserted in place of the Lc cDNA.

EXAMPLE 2

PLANT CELL TRANSFORMATION

Mature dry seed of W22 A1 A2 Bz1 Bz2 C1 C2 r-g:Stadler B-b pl was obtained from Pioneer Hi-Bred International, Johnston, Iowa. Kernels were surface sterilized for 20 min in 20% Clorox/0.1% Tween-20, washed three times in sterile distilled water and allowed to imbibe for 14-18 hrs in distilled water at room temperature. After removing the pericarp, embryos were dissected out and germinated on MS medium solidified with 0.5% Gel-Rite. The remainder of the kernel was split into halves which were placed with the exposed aleurone layer upper most on MS plates containing 0.25 M sorbitol. Tissues were usually incubated at 30°C with an 18 hr/6 hr light-dark regimen. Half-kernels were bombarded the following day, whereas germinated seedlings were bombarded 36-48 hrs after plating. In most experiments, 5 ug each of pPHI459 and/or pPHI443 DNA was precipitated onto 4.375 mg of gold particles (Engelhard A1570 Flakeless) essentially as described in the patent application of Tomes, U.S. Serial No. 351,075, the disclosures of which are hereby incorporated herein by reference. Approximately 10^7 particles in a total volume of 1 uL were delivered using a particle gun as described by Sanford, et al. A single shot resulted in the most anthocyanin accumulating cells. Anthocyanin accumulation was first detectable at 14-18 hrs after bombardment in aleurone cells and some epidermal cells (e.g., scutellar node). In other tissues (e.g., epidermal cells of root, leaf, coleoptile, tassel, etc.), pigmentation often took 36-48 hrs to develop. In most cells, the red color was stable for several weeks following bombardment. Tissues were stained for GUS within 48 hrs following bombardment essentially as described by Jefferson et al., (1987) EMBO J., 6, 3901-3907, except that the staining solution contained 1% DMSO. All percentages herein are by weight unless otherwise indicated. Blue-stained cells containing GUS were usually visible within 2 hrs. If tissues containing red cells were left in the GUS stain for

longer than 8 hrs, the red color gradually started to fade over time.

Precipitation of 0.1-1.0 ug pPHI443 onto 4.375 mg of gold particles as described above was found to be optimal for aleurone cells. In one experiment, 100-500 red cells were obtained per half kernel using 0.5-1.0 ug pPHI443, and even with as little as 1 ng pPHI443 per 4.375 mg aliquot of particles, a few red cells were observed on each aleurone. A typical dose-response curve is shown in Fig. 1. Since Lc does not normally condition pigmentation in the aleurone cell layer, this demonstrates that Lc is functionally equivalent to the S gene. Thus, by replacing the normal Lc promoter with signals which promote transcription, pigmentation can be induced in tissues where Lc does not normally act.

Industrial Applicability

R locus gene expression in plant tissues is conditioned by the Pl locus. In the absence of Pl, anthocyanin biosynthesis is light-dependent in all tissues except for the aleurone layer. Pigmentation in the aleurone layer requires Cl, which is functionally equivalent to Pl. However, when Cl Pl and Cl pl aleurones and seedling tissues which had been incubated in either the light or the dark were bombarded with pPHI443, it was determined that aleurone pigmentation resulting from the introduction of pPHI443 was independent of either Pl or light, whereas seedling pigmentation required one or the other of these factors. This characteristic provides the ability to distinguish between stably and transiently transformed cells by incubating the cells in the dark for several days post-bombardment and then returning them to growth in the light. Only cells which have been stably transformed will then become pigmented.

Following bombardment of germinated seedlings, pigmentation was seen in the epidermal layer of the

coleoptile, mesocotyl, scutellar node, coleorhiza and both primary and adventitious roots. In other experiments, pigmentation was frequently observed in the trichomes and marginal hairs of young leaves and tassel glumes, as well as in epidermal and sub-epidermal cells. Red cells were also seen at lower frequency in the inner and outer layers of immature pericarp, immature tassels and anther locules and both culm and husk tissues. Surprisingly, pigmented cells were never observed in either immature or mature endosperm.

Many cells which express GUS following bombardment with pPHI459 exhibited a diffuse pattern of staining, suggesting that the GUS enzyme and/or the histochemical substrate may be leaking out as a result of damage induced by particle bombardment. In contrast, anthocyanins leak out of cells very quickly if the cell membrane is perturbed, so that those cells which are damaged by bombardment will not accumulate pigment even if the marker gene is expressed. This offers an advantage in distinguishing between transformed damaged cells and transformed intact cells.

Cell-autonomous expression can be visualized in almost all tissues of the plant without having to disturb the integrity of the plant. This is extremely useful for developmental studies of maize and other plants and for obtaining stably transformed plants. By introducing the gene into meristematic cells, pigmented sectors of tissues can be followed during subsequent development. Stably transformed sectors which give rise to germ line tissues can yield transformed plants in the following generation.

This invention also provides materials and methods for the creation of new ornamental plants in which selected cells or tissues, or all cells or tissues, produce and accumulate anthocyanin pigmentation.

WHAT IS CLAIMED IS:

1. An expression cassette comprising a DNA sequence coding for a transcriptional activator for anthocyanin biosynthesis operably linked to plant regulatory sequences which cause the expression of the DNA sequence in plant cells.

2. An expression cassette according to claim 1 wherein the DNA sequence is a genomic or cDNA clone or translational equivalent thereof.

3. An expression cassette according to Claim 2 wherein the sequence codes for a maize R gene or a translational equivalent thereof.

4. An expression cassette according to Claim 3 wherein the gene is the maize Lc gene.

5. A bacterial transformation vector comprising an expression cassette according to Claim 1, operably linked to bacterial expression regulatory sequences which cause replication of the expression cassette in bacterial cells.

6. Bacterial cells containing as a foreign plasmid at least one copy of a bacterial transformation vector according to Claim 5.

7. Transformed plant cells containing as foreign DNA at least one copy of the DNA sequence of an expression cassette according to Claim 1.

8. Transformed cells according to Claim 7, further characterized in being cells of a monocotyledonous species.

9. Transformed cells according to Claim 8, further characterized in being maize, sorghum, wheat, triticale or rice cells.

10. Transformed cells according to Claim 7, further characterized in being cells of a dicotyledonous species.

11. Transformed cells according to Claim 10, further characterized in being soybean, alfalfa, tobacco, canola or tomato cells.

12. A maize cell or tissue culture comprising cells according to claim 9.

13. A transformed maize plant, the cells of which contain as foreign DNA at least one copy of the DNA sequence of an expression cassette according to Claim 1.

14. A method of plant transformation using anthocyanin as a non-phytotoxic marker, comprising the steps of:

a) culturing cells or tissues from a selected target plant,

b) introducing into the cells of the cell or tissue culture at least one copy of an expression cassette comprising a DNA sequence coding for a transcriptional activator for anthocyanin biosynthesis operably linked to plant regulatory sequences which cause the expression of the DNA sequence in the plant's cells, and

c) identifying transformed cells by their anthocyanin pigmentation.

15. A method according to Claim 14 in which transformed cells are separated from non-transformed cells by a cell sorter which sorts on the basis of color.

16. A method according to Claim 14 in which the expression cassette is introduced into the cells by electroporation.

17. A method according to Claim 14 in which the expression cassette is introduced into the cells by microparticle bombardment.

18. A method according to Claim 14 in which the expression cassette is introduced into the cells by microinjection.

19. A method according to Claim 14 for transformation of Agrobacterium tumefaciens-susceptible dicotyledonous plants in which the expression cassette is introduced into the cells by infecting the cells with Agrobacterium tumefaciens, a plasmid of which has been modified to include the expression cassette.

20. A method of plant transformation to produce plants having ornamental anthocyanin coloration, comprising the steps of:

a) culturing cells or tissues from a selected target plant,

b) introducing into the cells of the cell or tissue culture at least one copy of an expression cassette comprising a DNA sequence coding for a transcriptional activator for anthocyanin biosynthesis, operably linked to plant regulatory sequences which cause the expression of the DNA sequence in the plant's cells, and

c) regenerating whole plants from the transformed cells.

1/3


RED CELL NUMBER
0
100
200
300
400
500
600
0.001
0.005
0.01
0.05
0.1
0.5
1.0
5.0
10.0
µg pPHI443

Figure 1

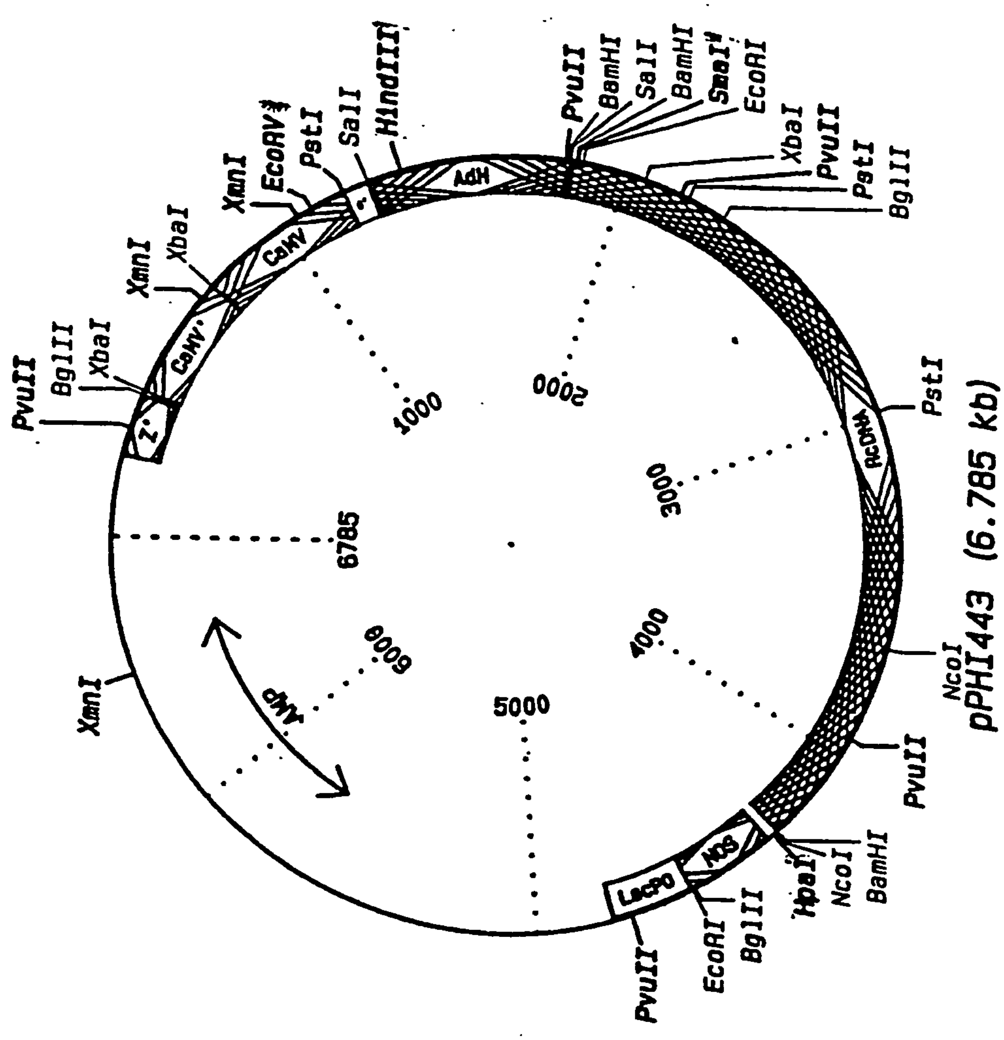


Figure 2

PLASMIDMAP of: Pphi443.Seq check: 1664 from: 1 to: 6785

Simsl April 18, 1989 09:59

```
-140 cccaaggttcgtggcatatctgtaggcatctaccccgtcttcgtcgtccgctcctcactagctaccaagggtcgccattattgccaacatagagtgtacgtggatgtctatatatatgcctacttgcacccatatggca

   1 TAGGCGTTCGATCCCCTTAGCGCGGAGGAGAGCTCCTCCGGTTCTTCTCTACCCTTCGCATGGAAGTTCTTGCATTGCTTCGTTGCTTCTCTAGTTTCTTCCTTCTACGTCTTTCCAGCATACGCATGCCCCTCGTCCGC

                                                                                                     M  A  L  S  A  S  R  V  Q  Q  A  E  E  L  L
 141 CGGTTCACGAGGCATCGTCTGATGATCAGTAGATAATAAGCAATATAATACTGATCTAGAATCGAGTTGTTGTACTCTTCGCAGATAGGCGCGTGATGGCGCTTTCAGCTTCCCGAGTTCAGCAGGCGGAAGAACTGCTG
      Q  R  P  A  E  R  Q  L  M  R  S  Q  L  A  A  A  A  R  S  I  N  W  S  Y  A  L  F  W  S  I  S  D  T  Q  P  G  V  L  T  W  T  D  G  F  Y  N  G
 281 CAACGACCTGCTGAGAGGCAGCTGATGAGGAGCCAGCTTGCTGCAGCCGCCAGGAGCATCAACTGGAGCTACGCCCTCTTCTGGTCCATTTCAGACACTCAACCAGGGGTGCTGACGTGGACGGACGGGTTCTACAACGG
       E  V  K  T  R  K  I  S  N  S  V  E  L  T  S  D  Q  L  V  M  Q  R  S  D  Q  L  R  E  L  Y  E  A  L  L  S  G  E  G  D  R  R  A  A  P  A  R
 421 CGAGGTGAAGACGCGGAAGATCTCCAACTCCGTGGAGCTGACATCCGACCAGCTCGTCATGCAGAGGAGCGACCAGCTCCGGGAGCTCTACGAGGCCCTCCTGTCGGGCGAGGGCGACCGCCGCGCTGCGCCTGCGCGGC
      P  A  G  S  L  S  P  E  D  L  G  D  T  E  W  Y  Y  V  V  S  M  T  Y  A  F  R  P  G  Q  G  L  P  G  R  S  F  A  S  D  E  N  V  W  L  C  N  A
 561 CGGCCGGCTCTCTGTCGCCGGAGGACCTCGGCGACACCGAGTGGTACTACGTGGTCTCCATGACCTACGCCTTCCGGCCAGGCCAAGGGTTGCCCGGCAGGAGTTTCGCCAGCGACGAGAACGTGTGGCTGTGCAACGCG
      H  L  A  G  S  K  A  F  P  R  A  L  L  A  K  S  A  S  I  Q  S  I  L  C  I  P  V  N  G  G  V  L  E  L  G  T  T  D  T  V  P  E  A  P  D  L  V
 701 CACCTCGCCGGCAGCAAAGCCTTCCCCCGCGCGCTCCTGGCCAAGAGCGCGTCCATTCAGTCAATCCTCTGCATCCCGGTTAACGGCGGCGTGCTTGAGCTTGGTACAACTGACACGGTGCCGGAGGCCCCGGACTTGGT
      S  R  A  T  A  A  F  W  E  P  Q  C  P  S  S  S  P  S  G  R  A  N  E  T  G  E  A  A  A  D  D  G  T  F  A  F  E  E  L  D  H  N  N  G  M  D
 841 CAGCCGAGCCACCGCGGCCTTCTGGGAGCCGCAGTGCCCGAGCTCCAGCCCGTCAGGACGAGCAAACGAGACCGGCGAGGCCGCAGCAGACGACGGCACGTTTGCGTTCGAGGAACTCGACCACAATAATGGCATGGACG
      D  I  E  A  M  T  A  A  G  G  H  G  Q  E  E  E  L  R  L  R  E  A  E  A  L  S  D  D  A  S  L  E  H  I  T  K  E  I  E  E  F  Y  S  L  C  D  E
 981 ACATAGAGGCGATGACCGCCGCCGGCGGCCACGGGCAGGAGGAGGAGCTAAGACTAAGAGAAGCCGAGGCCCTGTCAGACGACGCCAGCCTGGAGCACATCACCAAGGAGATCGAGGAGTTCTACAGCCTCTGCGACGAA
      M  D  L  Q  A  L  P  L  P  L  E  D  G  W  T  V  D  A  S  N  F  E  V  P  C  S  S  P  Q  P  A  P  P  P  V  D  R  A  T  A  N  V  A  A  D  A  S
1121 ATGGACCTGCAGGCGCTACCACTACCGCTAGAGGACGGCTGGACCGTGGACGCGTCCAATTTCGAGGTCCCCTGCTCTTCCCCGCAGCCAGCGCCGCCTCCGGTGGACAGGGCTACCGCTAACGTCGCCGCCGACGCCTC
      R  A  P  V  Y  G  S  R  A  T  S  F  H  A  W  T  R  S  S  Q  Q  S  S  C  S  D  D  A  A  P  A  A  V  V  P  A  I  E  E  P  Q  R  L  L  K  K
1261 AAGGGCACCCGTCTACGGCTCTCGGGCGACGAGTTTCATGGCTTGGACCAGGTCCTCGCAGCAGTCGTCGTGCTCCGACGACGCGGCGCCCGCAGCAGTAGTGCCGGCCATCGAGGAGCCGCAGAGATTGCTGAAGAAAG
      V  V  A  G  G  G  A  W  E  S  C  G  G  A  T  G  A  A  Q  E  N  S  G  T  G  T  K  N  H  V  M  S  E  R  K  R  R  E  K  L  N  E  M  F  L  V  L
1401 TGGTGGCCGCCGGCGGTGCTTGGGAGAGCTGTGGCGGCGCCACGGGCGCAGCACAGGAAAATAGTGGCACTGGCACCAAGAACCACGTCATGTCGGAGCGAAAGCGACGAGAGAAGCTCAACGAGATGTTCCTCGTCCTC
      K  S  L  L  P  S  I  H  R  V  N  K  A  S  I  L  A  E  T  I  A  Y  L  K  E  L  Q  R  R  V  Q  E  L  E  S  S  R  E  P  A  S  R  P  S  E  T  T
1541 AAGTCACTGCTTCCGTCCATTCACAGGGTGAACAAAGCGTCGATCCTCGCCGAAACGATAGCCTACCTCAAGGAGCTTCAGAGAAGGGTGCAAGAGCTGGAGTCCAGTAGGGAACCTGCGTCGCGCCCATCCGAAACGAC
      T  R  L  I  T  R  P  S  R  G  N  N  E  S  V  R  K  E  V  C  A  G  S  K  R  K  S  P  E  L  G  R  D  D  V  E  R  P  P  V  L  T  M  D  A  G
1681 GACAAGGCTAATAACAAGGCCGTCCCGTGGCAATAATGAGAGTGTGAGGAAGGAGGTCTGCGCGGGCTCCAAGAGGAAGAGCCCAGAGCTCGGCAGAGACGACGTGGAGCGCCCCCCGGTCCTCACCATGGACGCCGGCA
      T  S  N  V  T  V  T  V  S  D  K  D  V  L  L  E  V  Q  C  R  W  E  E  L  L  M  T  R  V  F  D  A  I  K  S  L  H  L  D  V  L  S  V  Q  A  S  A
1821 CCAGCAACGTCACCGTCACCGTCTCGGACAAGGACGTGCTCCTGGAGGTGCAGTGCCGGTGGGAGGAGCTCCTGATGACGCGAGTGTTCGACGCCATCAAGAGCCTCCATTTGGACGTCCTCTCGGTTCAGGCTTCAGCG
      P  D  G  F  M  G  L  K  I  R  A  Q  F  A  G  S  G  A  V  V  P  W  M  I  S  E  A  L  R  K  A  I  G  K  R  -
1961 CCAGATGGCTTCATGGGGCTTAAGATACGAGCTCAGTTTGCTGGCTCCGGTGCCGTCGTGCCCTGGATGATCAGCGAGGCTCTTCGCAAAGCTATAGGGAAGCGGTGAAGGGGCAGCTGGAAATTTGGACATCGACGGCG

2101 ATGGAAGGCTTCATGGGATCGAAGCAAAGATGTATTTCTTGTTTCTTTAGATAACAGACATGAATCGGACCTTTTATATCAACAATTATATGGGCATGAATACTTAAGACTCCAGCCCTTAACACGTAGAAACTCAAAAAA

2241 GAAGACAGGAAGCTAAAGACTAAGCGTAAGGTATATTTGGAAGTAAATTATTTTTATAGTTTCTAAGCAATCTCATGGTTTATAGGAATACTAGAGTGTTTATGGCATAAGGTGTTTGGTTGCATTCATAAAACCTATAT

2381 TTTCAAAGTCATAGCATTCTAGATACCATGATATTTTTGTAATATTGGAAACTACACTCCAACGCAAAGTTTTTATGACATGGCT
```

Figure 3

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/04281

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 15/00; C12N 5/00; C12N 1/20; C12N 1/00, A01H 5/00
U.S.Cl.: 435/172.1,172.3,240.4,252.3, ,320; 800/205

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/172.1,172.3,240.4,252.3,317.1,320 800/205 935/35,64,67

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[6]

USPTO AUTOMATED PATENT SYSTEM; DIALOG Files: BIOTECH, PATENTS, WORLD PATENTS INDEX. See Attachment for Search Terms.

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]

Category[*]	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
X Y	The EMBO Journal, Volume 6, No. 12, Issued 1987, (Oxford England) Paz-Ares et al, "The regulatory cl locus of Zea mays encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcriptional activators", pages 3553-3558. (See entire document).	1-3,5-6 1-3,5-20
X Y	Chromosome Structure and Function; Gustafson et al. (eds) Issued 1988; Plenum Press (New York, USA); Dellaporta et al.; "Molecular Cloning Of The Maize R-nj Allele By Transposon Tagging With Ac", pages 263-282. (See entire document).	1-3,5-6 1-3,5-20

* Special categories of cited documents:[15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search[2]	Date of Mailing of this International Search Report[2]
23 October 1990	18 JAN 1991
International Searching Authority[1]	**Signature of Authorized Officer**[20]
ISA/US	P. Rhodes, Examiner

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Proceedings of the National Academy of Sciences USA; Volume 85; Issued June 1988; Klein et al.; "Transfer of foreign genes into intact maize cells with high-velocity microprojectiles"; pages 4305-4309. (See entire document).	1-3,5-14, and 17
Y	Nature; Volume 330 Issued 17 December 1987; (London, England); Meyer et al.; "A new petunia flower colour generated by transformation of a mutant with a maize gene", pages 677-678. (See entire document).	1-3,5-20
Y	US, A, 4,833,080 (Brent et al.) 23 May 1989, see entire document.	1-3,5-19
Y	US, A, 4,771,002 (Gelvin) 13 September 1988, see entire document.	1-3,5-20
Y	US, A, 4,300,310 (Galbraith)17 November 1981, see entire document.	14-15
Y	US, A, 4,649,109 (Perlman) 10 March 1987, see entire document.	14-15
Y	Physiologia Plantarum; Volume 68; Issued 1986; (London, England); Perani et al.; "Gene transfer methods for crop improvement: Introduction of foreign DNA into plants"; pages 566-570. See entire document.	14,16,18-19
P,Y	Nucleic Acids Research, Volume 17, Issued October 1989, (Oxford, England), Perrat et al., "Nucleotide sequence of the maize R-S gene", page 8003. See entire document.	1-13
P,X	Science; Volume 247; Issued 26 January 1990; Ludwig et al.; "A regulatory gene as a novel visible marker for maize transformation", pages 449-450. See entire document.	1-20

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

P,X	Proceedings of the National Academy of Sciences USA; Volume 86, Issued September 1989; Ludwig et al; "Lc, a member of the maize R gene family responsible for tissue-specific anthocyanin production, encodes a protein similar to transcriptional activators and contains the myc-homology region"; pages 7092-7096. (See entire document).	1-6

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter[1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out[1], specifically:

3. ☐ Claim numbers__________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this International application as follows:

(See Attachment).

1. ☒ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application. Telephone Practice.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the International application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment to Form PCT/ISA/210, Part II.

II. FIELDS SEARCHED SEARCH TERMS:

anthocyanin, R locus, paramutation, transcriptional activator, maize, transposable